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Cyclopiazonic Acid Is a Specific Inhibitor of the Ca^{2+}-ATPase of Sarcoplasmic Reticulum*

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The mycotoxin, cyclopiazonic acid (CPA), inhibits the Ca^{2+}-stimulated ATPase (EC 3.6.1.38) and Ca^{2+} transport activity of sarcoplasmic reticulum (Goeger, D. E., Riley, R. T., Dorner, J. W., and Cole, R. J. (1988) *Biochem. Pharmacol.* 37, 978–981). We found that at low ATP concentrations (0.5–2 μM) the inhibition of ATPase activity was essentially complete at a CPA concentration of 6–8 nmol/mg protein, indicating stoichiometric reaction of CPA with the Ca^{2+}-ATPase. Cyclopiazonic acid caused similar inhibition of the Ca^{2+}-stimulated ATP hydrolysis in intact sarcoplasmic reticulum and in a purified preparation of Ca^{2+}-ATPase. Cyclopiazonic acid also inhibited the Ca^{2+}-dependent acetylphosphate, *p*-nitrophenylphosphate and carbamylphosphate hydrolysis by sarcoplasmic reticulum. ATP protected the enzyme in a competitive manner against inhibition by CPA, while a 10^5-fold change in free Ca^{2+} concentration had only moderate effect on the extent of inhibition. CPA did not influence the crystallization of Ca^{2+}-ATPase by vanadate or the reaction of fluorescein-5′-isothiocyanate with the Ca^{2+}-ATPase, but it completely blocked at concentrations as low as 1–2 mol of CPA/mol of ATPase the fluorescence changes induced by Ca^{2+} and [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) in FITC-labeled sarcoplasmic reticulum and inhibited the cleavage of Ca^{2+}-ATPase by trypsin at the T_2 cleavage site in the presence of EGTA. These observations suggest that CPA interferes with the ATP-induced conformational changes related to Ca^{2+} transport.

The effect of CPA on the sarcoplasmic reticulum Ca^{2+}-ATPase appears to be fairly specific, since the kidney and brain Na^+,K^+-ATPase (EC 3.6.1.37), the gastric H^+,K^+-ATPase (EC 3.6.1.36), the mitochondrial F_1-ATPase (EC 3.6.1.34), the Ca^{2+}-ATPase of erythrocytes, and the Mg^{2+}-activated ATPase of T-tubules and surface membranes of rat skeletal muscle were not inhibited by CPA, even at concentrations as high as 1000 nmol/mg protein.

Fungal toxins are common contaminants in agricultural products that present potential health risks to farm animals and humans. Cyclopiazonic acid (CPA),[1] an indole tetramic acid metabolite of *Aspergillus* and *Penicillium*, is one such mycotoxin that produces toxic effects in muscle (1, 2). Goeger and his colleagues (3) found that CPA is a potent inhibitor of the Ca^{2+} uptake and ATPase activity of rat skeletal muscle sarcoplasmic reticulum (SR) and suggested that this inhibitory action may be involved in CPA toxicity.

The Ca^{2+}-ATPase is a major protein component of the SR membrane that regulates the contraction-relaxation cycle in muscle by transport of Ca^{2+} from the cytoplasm into the lumen of the SR, coupled to the hydrolysis of ATP. The primary sequence of the Ca^{2+}-ATPase has been determined (4), two stable conformations of the enzyme have been crystallized (5–9), and mapping of the various functional domains has begun (10). The kinetic mechanism of Ca^{2+}-dependent ATP hydrolysis was determined in considerable detail (11) but so far no specific, high affinity inhibitor of the enzyme has been identified.

We further examined the reaction of CPA with the Ca^{2+}-ATPase of sarcoplasmic reticulum by kinetic analysis of its effects on the hydrolysis of ATP, acetylphosphate, *p*-nitrophenylphosphate, and carbamylphosphate and by testing its influence on the conformational dynamics of the Ca^{2+}-ATPase. We find that CPA specifically interferes with the conformational transitions of the Ca^{2+}-ATPase without significant effect on the hydrolysis of ATP by the Na^+,K^+-ATPase, the H^+,K^+-ATPase, the mitochondrial F_1-ATPase, the erythrocyte Ca^{2+}-ATPase, and the Mg^{2+}-activated ATPase of muscle cell surface membranes. Therefore, CPA may serve as a specific high affinity inhibitor of the sarcoplasmic reticulum Ca^{2+} pump. A preliminary report of some of these results has been presented (12).

EXPERIMENTAL PROCEDURES

Materials

Cyclopiazonic acid, acetylphosphate, *p*-nitrophenylphosphate, disodium-ATP, Tris-ATP, EGTA, Tris, NADH, imidazole, phosphoenolpyruvate kinase (rabbit muscle), lactate dehydrogenase (rabbit muscle), trypsin (bovine pancreas), trypsin inhibitor (soybean), acrylamide, bisacrylamide, ammonium persulfate, albumin (bovine serum), Tris-maleate, MOPS, and Me_2SO were supplied by Sigma. Sodium vanadate, hydroxylamine hydrochloride and 2-mercaptoethanol were purchased from Fisher. Sodium dodecyl sulfate was obtained from Polysciences, Inc., Washington, PA; TEMED from Eastman Organic Chemicals, Rochester, NY; Coomassie Brilliant Blue R-250 from Bio-Rad; and molecular weight markers from Pharmacia LKB Biotechnology Inc. Fluorescein-5′-isothiocyanate was purchased from Molec-

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[1] The abbreviations are: CPA, cyclopiazonic acid; SR, sarcoplasmic reticulum; FITC, fluorescein-5′-isothiocyanate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

EXHIBIT A

ular Probes, Inc., Eugene, OR; Ca^{2+} ionophore A23187 and carbamylphosphate were from Behring Diagnostics. All other chemicals were of analytical grade.

Isolation of Transport ATPases

SR vesicles were obtained by differential centrifugation of homogenates of predominantly white, rabbit skeletal muscle (13, 14). The purified Ca^{2+}-ATPase was prepared according to Meissner *et al.* (15).

A rat skeletal muscle surface membrane and T-tubule preparation was isolated, and its Mg^{2+}-ATPase activity was assayed according to Beeler *et al.* (16, 17). The preparation was kindly provided to us by Dr. Troy J. Beeler of the Dept. of Biochemistry, Uniformed Services University of the Health Sciences, Bethesda.

H^+,K^+-ATPase prepared from pig gastric mucosa as described by Ray *et al.* (18) was kindly supplied by Dr. Tushar K. Ray of the Department of Surgery, SUNY Health Science Center at Syracuse.

Na^+,K^+-ATPase purified from dog kidney according to Jorgensen (19), was given to us by Dr. Joseph D. Robinson, Jr., of the Department of Pharmacology, SUNY Health Science Center at Syracuse. The Na^+,K^+-ATPase and Mg^{2+}-ATPase activities were also tested in rabbit brain microsome preparations isolated according to Hart and Titus (20).

Mitochondrial F_1-ATPase prepared from beef heart according to Penefsky (21) was provided by Dr. Harvey S. Penefsky of the Department of Biochemistry and Molecular Biology, SUNY Health Science Center at Syracuse.

The human erythrocyte surface membrane Ca^{2+}-ATPase was purified according to Niggli *et al.* (22) and was kindly sent to us by Dr. John T. Penniston, Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota.

Assay of the Sarcoplasmic Reticulum and Erythrocyte Surface Membrane Ca^{2+}-ATPases

The rate of Ca^{2+}-dependent ATP hydrolysis was determined at various concentrations of Ca^{2+}, ATP, and CPA, either by a coupled-enzyme system (14, 23) or by analysis of inorganic phosphate (P_i) release according to the method of Fiske and Subbarow (24).

The composition of the final reaction mixture for the coupled enzyme assay was 0.1 M KCl, 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, ±0.7 mM $CaCl_2$, 0.5 μM to 5.0 mM ATP, 0.5 mM EGTA, 0.42 mM phosphoenolpyruvate, 0.2 mM NADH, 7.5 IU/ml of pyruvate kinase, 18 IU/ml of lactic acid dehydrogenase, 1–5 μg of protein/ml, 1–2 μM A23187 and 0–1000 nmol CPA/mg SR protein. The enzymes were preincubated with CPA for 5 min at 25 °C prior to the start of the reaction with the addition of ATP-containing medium. The change in absorbance was measured at 340 nm at 25 °C using a Perkin-Elmer Lambda 3B spectrophotometer. The recorded absorbance was linear up to at least 5–10 min of reaction time. The Ca^{2+}-insensitive ATP hydrolysis measured with Ca^{2+} omitted from the medium was usually less than 5% of the rate observed at optimal Ca^{2+} concentration. The rate of Ca^{2+}-stimulated ATP hydrolysis was obtained as the difference between the total and Ca^{2+}-insensitive hydrolysis rates.

The rate of inorganic phosphate liberation from ATP was determined by incubation of SR vesicles (0.25 mg of protein/ml) at 25 °C in an assay medium of 0.05 M KCl, 0.05 M imidazole, pH 7.0, 5 mM $MgCl_2$, 0–2 mM $CaCl_2$, 1 mM EGTA, 2 μM A23187, 1 mM ATP, and cyclopiazonic acid at concentrations ranging between 0 and 100 nmol/mg protein.

Systems of identical composition were used for the assay of the Ca^{2+}-ATPase of sarcoplasmic reticulum in the native membrane and in purified preparations, and of the Ca^{2+}-ATPase of erythrocyte membrane.

Protein was determined according to Lowry *et al.* (25).

Assays of H^+,K^+-ATPase, Na^+,K^+-ATPase, and Mitochondrial F_1-ATPase

The phosphohydrolase activities of H^+,K^+-ATPase, Na^+,K^+-ATPase, and mitochondrial F_1-ATPase were measured at 25 °C in coupled-enzyme assay systems of suitable composition. The final composition of the reaction systems for the various enzymes was as follows.

H^+,K^+-ATPase—20 mM HEPES/Tris, pH 6.8, 2 mM $MgCl_2$, 0.2–2 mM Tris-ATP, 0.42 mM phosphoenolpyruvate, 0.2 mM NADH, 7.5 IU/ml of pyruvate kinase and 18 IU/ml of lactate dehydrogenase, with or without 20 mM KCl. The enzyme vesicles were permeabilized by treatment with 0.01% sodium dodecyl sulfate for 30 min at 25 °C prior to addition to the assay system at a final concentration of 10 μg protein/ml. The activity measured in the absence of K^+ and Mg^{2+} was deducted from the activity in the complete assay system to obtain the specific activity of H^+,K^+-ATPase.

Na^+,K^+-ATPase—The assay media contained in final concentration 30 mM KCl, 20 mM HEPES/Tris, pH 7.5, 3.5 mM $MgCl_2$, 0.3–3 mM Tris-ATP, 0.1 mM Tris-EGTA, 0.42 mM phosphoenolpyruvate, 0.2 mM NADH, 7.5 IU/ml of pyruvate kinase, and 18 IU/ml of lactate dehydrogenase, with or without 120 mM NaCl. The final concentration of enzyme was 5 μg/ml. The Na^+,K^+-ATPase activity was calculated by subtracting the activity obtained in the absence of Na^+ from the activity measured in the complete assay system. In the analysis of the Na^+,K^+-ATPase activity of brain microsomes, the contribution of Na^+,K^+-ATPase to the total Mg^{2+}-stimulated ATPase activity was also assessed by testing the inhibition caused by 0.1 mM ouabain.

Mitochondrial F_1-ATPase—The medium contained in final concentrations 50 mM Tris-SO_4, pH 8.0, 6 mM $MgCl_2$, 0.6–6 mM ATP, 0.42 mM phosphoenolpyruvate, 0.2 mM NADH, 7.5 IU/ml of pyruvate kinase and 18 IU/ml of lactate dehydrogenase. The final concentration of enzyme was 0.5 μg/ml. In this case preincubation of enzyme with CPA was limited to 1 min prior to start of the reaction with ATP, in view of the lability of the enzyme. There was no indication of inhibition by CPA in assays extended over a 6-min period.

The Mg^{2+}-activated ATPase of Skeletal Muscle Surface Membrane—The Mg^{2+}-stimulated ATPase activity was measured at 25 °C at pH 6.8 or 7.8 in a medium of 0.15 M KCl, 10 mM histidine, 5 mM $MgSO_4$, 4 mM EGTA, 2 mM ATP, 2 μM A23187, 0.42 mM phosphoenolpyruvate, 0.2 mM NADH, 7.5 IU/ml of pyruvate kinase, 18 IU/ml of lactate dehydrogenase, and 25 μg/ml of wheat germ agglutinin. The protein concentration was 1 μg/ml. In parallel assays the EGTA was replaced by 0.1 mM $CaCl_2$ to assess the possible contribution of the sarcoplasmic reticulum Ca^{2+}-ATPase. The ATPase preparation was preincubated with 0, 100, 1,000, and 10,000 nmol of CPA/mg protein for 5 min prior to the addition to the assay system.

The Hydrolysis of Substrate Analogs by the SR Ca^{2+}-ATPase

The acetylphosphatase and carbamylphosphatase activities were measured as described by Pucell and Martonosi (26). The acetylphosphate was determined by the method of Lipmann and Tuttle (27) and carbamylphosphate according to Spector *et al.* (28). The hydrolysis of *p*-nitrophenylphosphate by SR was analyzed as described by Varga *et al.* (14).

Other Measurements

The effects of pressure on CPA-treated SR microsomes were measured as described by Varga *et al.* (14). The labeling of the SR Ca^{2+}-ATPase with fluorescein-5′-isothiocyanate (FITC) and measurement of the effects of CPA on fluorescence intensity and polarization were done according to Papp *et al.* (29). The analysis of the effects of CPA on the vanadate-induced two-dimensional crystals of Ca^{2+}-ATPase involved methods described by Taylor *et al.* (9). The influence of CPA on the pattern of the tryptic cleavage of SR Ca^{2+}-ATPase was performed as in Ref. 14.

In all assays CPA was added in concentrated solutions in dimethyl sulfoxide; control samples received equal volumes of dimethyl sulfoxide without significant effects, either on the enzymatic activities or on structural properties of the Ca^{2+}-ATPase.

RESULTS AND DISCUSSION

The Effects of Cyclopiazonic Acid on the ATPase Activity of Sarcoplasmic Reticulum

With increasing concentration of cyclopiazonic acid (Fig. 1) we found progressive inhibition of the Ca^{2+}-dependent ATPase activity of sarcoplasmic reticulum (Fig. 2), in substantial agreement with earlier observations of Goeger and his colleagues (3). At low ATP concentrations (0.5–2.0 μM) significant inhibition of the Ca^{2+}-ATPase was obtained at CPA concentrations as low as 5–10 nmol/mg of sarcoplasmic reticulum protein, indicating nearly stoichiometric reaction of the CPA with the Ca^{2+}-ATPase; the inhibition became complete at ≈25 nmol of CPA/mg SR protein.

The dependence of the activity of Ca^{2+}-ATPase on ATP concentration is biphasic (30, 31). This results in a convex curvature of the double reciprocal plot of the steady state

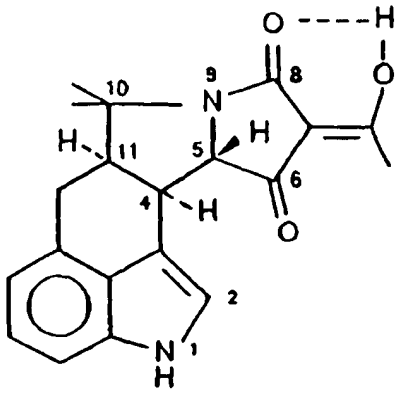

FIG. 1. **The structure of cyclopiazonic acid.**

ATPase activity at high ATP concentration (31), yielding two apparent K_m values of 2–3 and 500 μM, respectively. The inhibition of ATPase activity by CPA was particularly pronounced at low ATP concentration, causing sharp departure from linearity in the double reciprocal plot of ATPase hydrolysis (Fig. 2, *inset*). Increasing the concentration of ATP to 5 mM counteracted the effect of CPA, restoring the ATPase activity to near control levels (Fig. 2), suggesting competition. Dixon plots of the inhibitory effect of CPA at ATP concentrations of 0.5–5000 μM (Fig. 3) confirm the competitive relationship between CPA and ATP, with some indication of an irreversible component of inhibition at low ATP concentration. Due to the high affinity of the enzyme for CPA, the free CPA concentration for half-maximal inhibition by CPA could not be determined accurately, but it is estimated to be in the range of 10–20 nM. The intercept in the upper left quadrant of the Dixon plot corresponds to ≃6 nmol of CPA/mg SR protein, suggesting a nearly stoichiometric reaction of the CPA with the Ca^{2+}-ATPase. The inhibition of ATPase persisted after washing of the microsomes by repeated centrifugation, consistent either with tight binding of CPA to the enzyme in the absence of ATP or some modification of the Ca^{2+}-ATPase by CPA that persists after the removal of the inhibitor.

The Ca^{2+} transport ATPase of SR is activated by Ca^{2+} at micromolar concentrations, approaching maximum activity at ≃10^{-5} M free [Ca^{2+}] (31). The activity declines at millimolar Ca^{2+} concentrations due to inhibition of Ca^{2+} transport by high intravesicular Ca^{2+}. The inhibitory effect of CPA on the Ca^{2+}-ATPase was only moderately affected by changes in the Ca^{2+} concentration between 1–100 μM (Fig. 4).

The inhibitory effect of CPA on the Ca^{2+}-stimulated ATPase activity of purified Ca^{2+}-ATPase was similar to that seen in the native sarcoplasmic reticulum membrane. The specific activity of the purified Ca^{2+}-ATPase was 5.14 μmol·mg^{-1}·min^{-1} and it was reduced to 1.68 μmol·mg^{-1}·min^{-1} in the presence of 100 nmol of CPA/mg protein in an assay system containing 5 mM ATP. Essentially similar inhibition was obtained after reconstitution of Ca^{2+}-ATPase into vesicular structures by removal of detergent through dialysis.

The Effect of CPA on the Hydrolysis of Acetylphosphate, p-Nitrophenylphosphate, and Carbamylphosphate by the Sarcoplasmic Reticulum Ca^{2+}-ATPase

The apparent competition between CPA and ATP points to the possible involvement of the nucleotide-binding domain in the inhibition of ATPase activity by CPA. This possibility was tested by measuring the effect of CPA on the hydrolysis of acetylphosphate, *p*-nitrophenylphosphate, and carbamylphosphate by the sarcoplasmic reticulum. Although they lack the nucleotide ring, all three substrate analogs are cleaved by the Ca^{2+}-ATPase of SR and can serve as energy donors for Ca^{2+} transport (26, 31). As shown in Table I, the Ca^{2+}-dependent hydrolysis of acetylphosphate and *p*-nitrophenylphosphate was nearly completely inhibited by 40–100 nmol of CPA/mg SR protein, while the Ca^{2+}-insensitive component of the hydrolysis was relatively unaffected. At low CPA concentration (10 nmol/mg protein) significantly greater inhibition of the *p*-nitrophenylphosphatase activity was observed at low (≃10 μM) than at high (0.5 mM) Ca^{2+} concentration; this may be due to the formation of Ca^{2+}-CPA, since the difference diminished and eventually disappeared at higher CPA concentrations.

In contrast to ATP, acetylphosphate or *p*-nitrophenylphosphate, only about half of the carbamylphosphate hydrolysis is Ca^{2+} sensitive under the conditions shown in Table I. While the total carbamylphosphatase activity was apparently unaffected by CPA, the Ca^{2+}-insensitive component of carbamylphosphate hydrolysis was significantly elevated (Table I); the net result is an inhibition of the Ca^{2+}-stimulated carbamylphosphatase by CPA that is similar in magnitude to that

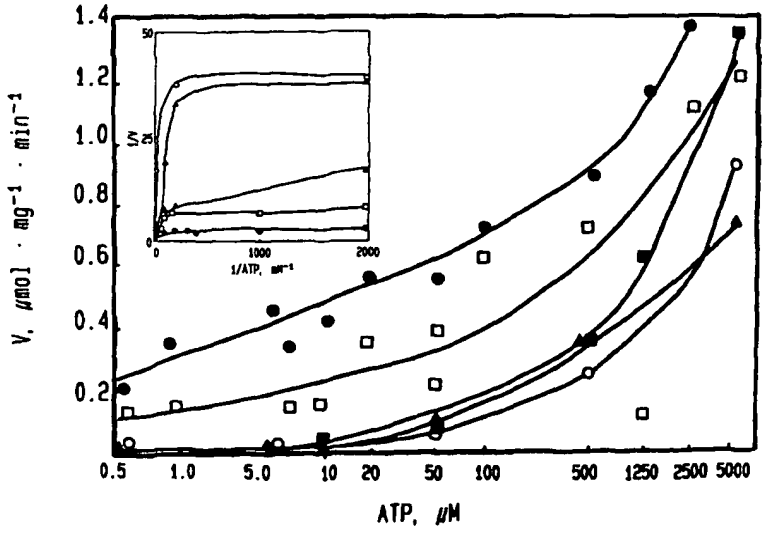

FIG. 2. **The effect of CPA on the Ca^{2+}-ATPase activity of sarcoplasmic reticulum.** The ATPase activity was measured using the coupled enzyme system as described under "Experimental Procedures." The reaction was started with ATP after preincubation of the enzyme with cyclopiazonic acid at the indicated concentration. The data are given as means of duplicate measurements from two to seven experiments using seven different sarcoplasmic reticulum preparations. *Inset*: double reciprocal plot of the same data. The final concentrations of CPA (nmol/mg protein) were as follows: ●, zero; □, 10; ■, 25; △, 30; ▲, 50; ○, 100.

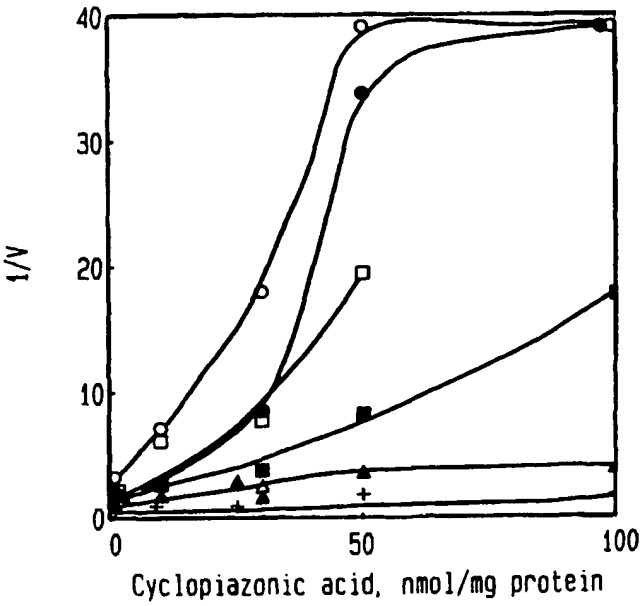

FIG. 3. **Dixon plots of SR Ca^{2+}-ATPase activity as a function of CPA and ATP concentrations.** Ca^{2+}-ATPase activity was measured at CPA concentrations of 0–100 nmol/mg protein. The concentrations of ATP (μM) were as follows: ○, 0.5; ●, 5.0; □, 10.0; ■, 50.0; △, 100.0; ▲, 500; +, 5000. Each data point represents the mean of duplicate assays from two to seven experiments on seven different sarcoplasmic reticulum preparations.

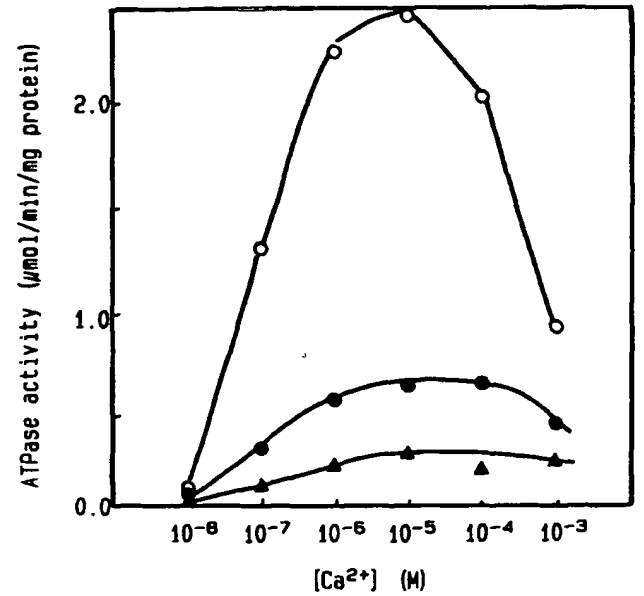

FIG. 4. **The effect of CPA on the Ca^{2+}-ATPase of sarcoplasmic reticulum at various concentrations of Ca^{2+}.** Ca^{2+}-ATPase activity was measured by the release of inorganic phosphate (P_i) from ATP, as described under "Experimental Procedures." The free [Ca^{2+}] was calculated from the relative amounts of Ca^{2+} and EGTA in the reaction mixture using an affinity constant of 10^6 M^{-1} for the Ca^{2+}-EGTA complex. The concentration of ATP was 1 mM. Control values, (○), are compared with values obtained in the presence of 30, (●), and 100, (▲), nmol CPA/mg protein.

observed with ATP, *p*-nitrophenylphosphate or acetylphosphate (Table I).

These observations indicate that the inhibitory effect of CPA, unlike that of fluorescein-5′-isothiocyanate, is not confined to nucleoside triphosphate substrates and therefore not likely to involve the nucleotide binding domain of the Ca^{2+}-ATPase.

The Effect of CPA on the Conformational Transitions of the Sarcoplasmic Reticulum Ca^{2+}-ATPase

CPA Effect on the Fluorescence of FITC-labeled Ca^{2+}-ATPase—FITC reacts with lysine 515 in the nucleotide-binding domain of the Ca^{2+}-ATPase, causing inhibition of ATPase activity; ATP protects the enzyme against reaction with FITC (32). The fluorescence of FITC-labeled ATPase decreases upon saturation of the high affinity Ca^{2+} sites of the Ca^{2+}-ATPase by Ca^{2+} (33) or by lanthanides (34), and increases in the presence of EGTA and vanadate. These changes in fluorescence intensity are taken to indicate a shift in the conformational equilibrium of Ca^{2+}-ATPase in favor of the E_1 state in the presence of Ca^{2+} or La^{3+}, and in favor of the E_2 state in the presence of EGTA and vanadate. The transition between the E_1 and E_2 states is an essential feature of Ca^{2+} transport (31).

Cyclopiazonic acid (6–120 nmol/mg protein) had no effect on the reaction of the Ca^{2+}-ATPase with FITC but completely inhibited the changes in the fluorescence intensity of FITC-labeled Ca^{2+}-ATPase caused by the addition of either Ca^{2+} or EGTA (Fig. 5). This effect was observed at concentrations as low as 6 nmol of CPA/mg protein, *i.e.* at 1:1 molar ratio with the Ca^{2+}-ATPase (Fig. 5). These observations imply that CPA inhibits the conformational transition of the enzyme between the E_1 and the E_2 states. Therefore, the inhibition of ATPase activity by CPA may be related to the inhibition of the conformational changes associated with ATP hydrolysis and Ca^{2+} transport. The polarization of fluorescence of FITC-labeled ATPase is relatively high ($P = 0.345$–0.351) indicating significant immobilization of the covalently bound FITC on the Ca^{2+}-ATPase (35). The polarization of fluorescence was not affected significantly by 1–100 nmol of CPA/mg protein in the presence of either 0.1 mM Ca^{2+} (E_1-state), or in a solution of 0.1 mM EGTA and 0.5 mM Na^+-orthovanadate containing mono- and oligovanadate anions (E_2-*V* state) (not shown).

The Effect of CPA on the Tryptic Proteolysis of Ca^{2+}-ATPase—The primary cleavage of the Ca^{2+}-ATPase by trypsin at the T_1 site (arginine 505) produces two large fragments (A and B) of nearly equal size (36). The secondary cleavage of fragment A by trypsin at the T_2 site (arginine 198) yields the A_1 and A_2 subfragments. Subsequent slower cleavage of the A_1, A_2, and B fragments produces a spectrum of smaller polypeptides. The cleavage at the T_1 site is not particularly sensitive to the conformation of the Ca^{2+}-ATPase, but the cleavage at the T_2 site is completely blocked by vanadate in the presence of EGTA (E_2-*V* state). This inhibition can be reversed by Ca^{2+} that converts the enzyme into the E_1 state (6, 7).

Cyclopiazonic acid (100 nmol/mg protein) had no effect on the cleavage of the Ca^{2+}-ATPase at the T_1 site. The rate of the subsequent cleavage of the A fragment at the T_2 site was significantly reduced by CPA in the presence of 1 mM EGTA (*i.e.* $\simeq 10^{-9}$ M Ca^{2+}) (Fig. 6, *C* and *D*), suggesting that a change in the conformation of the Ca^{2+}-ATPase caused by CPA limited the access of trypsin to the T_2 cleavage site.

Saturation of the high affinity binding sites of the Ca^{2+}-ATPase by $\simeq 10^{-5}$ M Ca^{2+} did not influence the inhibitory effect of CPA on the T_2 cleavage (not shown), but at high Ca^{2+} concentration (20 mM), the effect of CPA was no longer observed and the cleavage at the T_2 site proceeded at a fast rate with the formation of the A_1 and A_2 fragments (Fig. 6, *A*

TABLE I

Inhibition of acetylphosphate, p-nitrophenylphosphate, and carbamylphosphate hydrolysis by CPA

The acetylphosphatase and carbamylphosphatase activities were measured at 25 °C in a medium containing 0.1 M KCl, 10 mM imidazole, pH 7.0, 5 mM $MgCl_2$, 2 μM A23187, 0.25–0.5 mg of sarcoplasmic reticulum protein/ml, and either 0.1 mM EGTA (A) or 0.1 mM $CaCl_2$ (B). The reaction was started after 5 min preincubation with 0, 40, or 100 nmol of CPA/mg protein by the addition of 5 mM acetylphosphate or carbamylphosphate, respectively. The changes in acetylphosphate concentration were measured according to Lipmann and Tuttle (27). The liberation of inorganic phosphate from carbamylphosphate was measured according to Spector *et al.* (28). The liberation of *p*-nitrophenol from *p*-nitrophenylphosphate was followed spectrophotometrically at 420 nm as described earlier (14). The data are presented as the mean ± S.E. of 3–15 measurements made on 9 different sarcoplasmic reticulum preparations. The number of independent measurements made on each preparation varied. In the control samples (top line) each value represents the mean of 4–15 measurements; in the presence of 40 and 100 nmol of CPA/mg protein (middle and bottom lines) the number of independent assays was 3–6 and 3–9, respectively. The difference between the activities measured with and without Ca^{2+} (B-A) yields the Ca^{2+} dependent hydrolysis rate (C). Column D gives the % inhibition of the Ca^{2+}-dependent hydrolysis by cyclopiazonic acid.

CPA	Acetylphosphatase				Carbamylphosphatase				*p*-Nitrophenylphosphatase			
	A	B	C	D	A	B	C	D	A	B	C	D
nmol/mg	*μmol/mg/min*			%	*μmol/mg/min*			%	*nmol/mg/min*			%
None	0.10 ±0.01	0.42 ±0.02	0.32	0	0.30 ±0.02	0.66 ±0.04	0.36	0	5.09 ±0.75	27.50 ±3.85	22.41	0
40	0.12 ±0.01	0.10 ±0.02	−0.02	100	0.57 ±0.05	0.56 ±0.02	−0.01	100	5.21 ±0.86	7.51 ±0.18	2.30	90
100	0.02 ±0.02	0.09 ±0.01	0.07	78	0.65 ±0.03	0.73 ±0.06	0.08	78	4.98 ±0.64	6.27 ±0.83	1.29	95

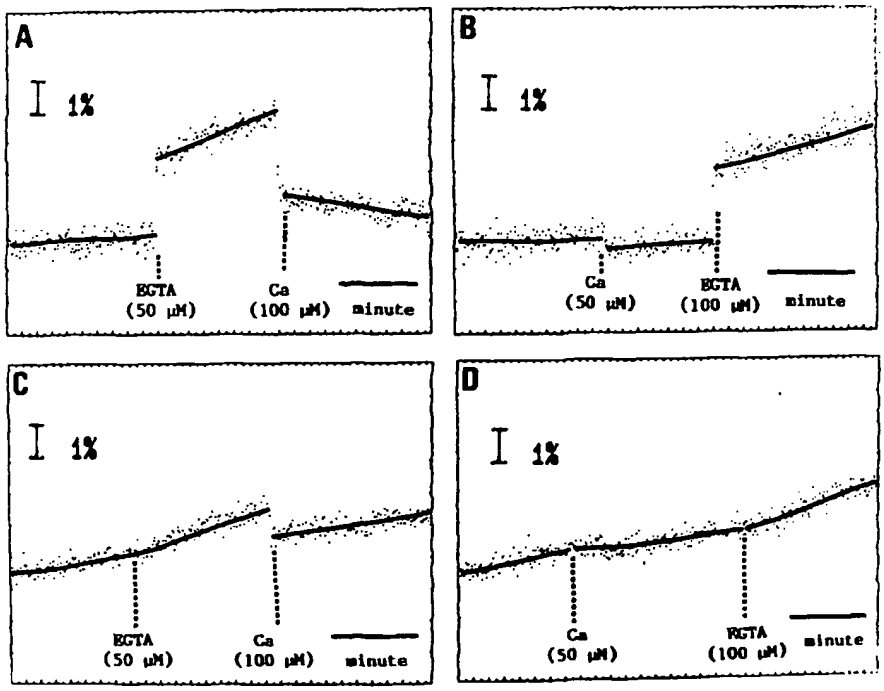

FIG. 5. **The effect of CPA on the fluorescence response of FITC-labeled Ca^{2+}-ATPase to Ca^{2+} and EGTA.** The Ca^{2+}-ATPase was labeled with FITC and the changes in fluorescence intensity caused by Ca^{2+} and EGTA were measured as described under "Experimental Procedures." The final concentrations of Ca^{2+} and EGTA are given in parentheses. A, B: no CPA; C, D: 6 nmol CPA/mg protein. CPA blocked the fluorescence intensity changes whether EGTA was added before (*A* and *C*) or after (*B* and *D*) the addition of Ca^{2+}. *Ordinate bars* indicate 1% change in fluorescence intensity.

and *B*). In the presence of 1 mM EGTA and 5 mM Na^+-orthovanadate, the enzyme is converted into the E_2-*V* state with complete inhibition of the T_2 cleavage, as observed earlier (6, 7); under these conditions CPA had no further effect (Fig. 6, *E* and *F*). The pattern of the cleavage of the B fragment, monitored by fluorescence after labeling the enzyme with FITC (37), was not significantly altered by CPA (not shown).

These observations suggest that CPA, similarly to vanadate, stabilizes a conformation of the Ca^{2+}-ATPase in which the tryptic cleavage of the A fragment into the A_1 and A_2 subfragments is inhibited.

Comparison of the Effects of CPA and Vanadate on the Crystallization and Pressure Sensitivity of Ca^{2+}-ATPase—Mono-, oligo-, and decavanadate anions in the presence of EGTA promote the formation of two-dimensional crystals of the Ca^{2+}-ATPase by stabilizing the E_2-*V* conformation (8, 9) and impart increased stability on the Ca^{2+}-ATPase during exposure to 1500–2000 atm pressure (14).

In contrast to vanadate, cyclopiazonic acid (20–200 nmol/mg protein) did not stimulate the formation of two-dimensional Ca^{2+}-ATPase crystals and did not protect or sensitize the Ca^{2+}-ATPase against pressure induced denaturation. Therefore, the mechanism of action of CPA does not involve the stabilization of the enzyme in a conformation similar to the state stabilized by vanadate. Neither did CPA (20–200 nmol/mg protein) disrupt the vanadate-induced crystals of Ca^{2+}-ATPase, that would be expected if CPA would shift the enzyme into a stable E_1-like conformation (7).

In summary, the studies with various conformational indicators suggest that CPA stabilizes the Ca^{2+}-ATPase in an

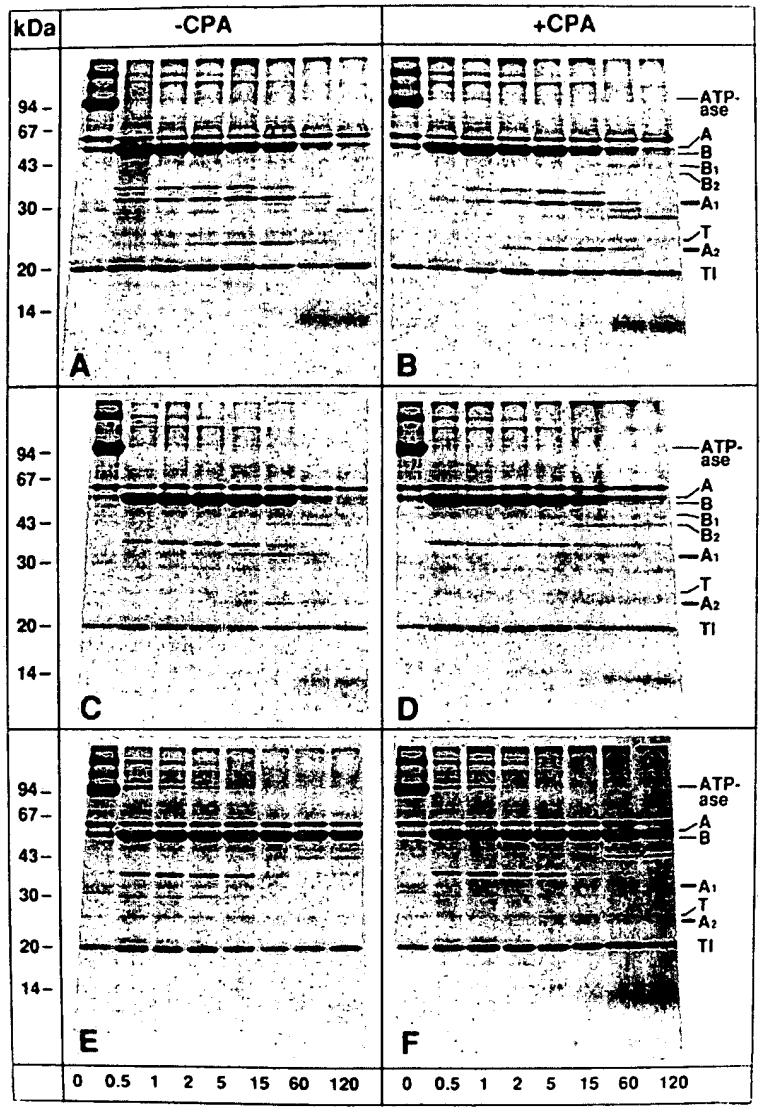

FIG. 6. **The effect of CPA on the tryptic digestion pattern of SR microsomes.** FITC-labeled SR vesicles (37) (2 mg protein/ml) were digested with trypsin (50 μg/ml) at 25 °C in a medium of 0.1 M KCl, 5 mM $MgCl_2$, 10 mM imidazole, pH 7.0, containing either 20 mM Ca^{2+} (*panels A* and *B*), or 1 mM EGTA (*panels C* and *D*), or 1 mM EGTA and 5 mM sodium vanadate (*panels E* and *F*) for times ranging from 0.5 to 120 min, as indicated on the *bottom line*. The digestion was carried out in the absence (*left panels A, C,* and *E*) or in the presence of 100 nmol of CPA/mg protein (*right panels B, D,* and *F*). The digestion was stopped by the addition of soybean trypsin inhibitor (100 μg/ml). To the zero time samples (O) trypsin and trypsin inhibitor were added together. The samples were solubilized in a solution of 5% sodium dodecyl sulfate, 10 mM Tris-HCl, pH 8.0, 1% 2-mercaptoethanol, 10% (v/v) glycerol, boiled at 100 °C for 10 min, and aliquots of 70 μg of protein were applied for electrophoresis on sodium dodecyl sulfate gradient gels (6–18%) as described in Ref. 6. The *far left column* indicates the positions of molecular weight markers in kilodalton (*kDa*). The *far right column* identifies band positions as follows: ATPase, Ca^{2+}-ATPase; A, B, A_1, A_2, B_1, and B_2, the corresponding proteolytic fragments of the Ca^{2+}-ATPase; T, trypsin; TI, trypsin inhibitor. The reaction of the enzyme with FITC had no influence on the course of tryptic digestion (37).

enzymatically inactive conformation that differs both from the E_1 state stabilized by Ca^{2+} and from the E_2 state stabilized by EGTA and vanadate.

The Specificity of CPA Effect on the Ca^{2+}-ATPase of Sarcoplasmic Reticulum

CPA inhibits the phosphorylation of Ca^{2+}-ATPase by ATP (38). Inhibition of some reaction step leading to enzyme phosphorylation is probably also involved in the inhibition by CPA of the hydrolysis of acetylphosphate, carbamylphosphate, and *p*-nitrophenylphosphate. Since phosphorylation of an active site aspartyl residue is a common feature of the P type ion transport ATPases, the effect of CPA on other ion transport enzymes was also explored.

The hydrolysis of ATP by the kidney Na^+,K^+-ATPase, the gastric H^+,K^+-ATPase, the Ca^{2+} transport ATPase of erythrocytes, and by the beef heart mitochondrial F_1 ATPase was not inhibited significantly by 100–1000 nmol of CPA/mg protein at either low (0.2–0.6 mM) or high (2–6 mM) ATP concentrations (Table II). The sarcoplasmic reticulum Ca^{2+}-ATPase tested under the specific conditions used for each of these enzymes was inhibited by 100 nmol of CPA to the extent of 59–89%.

For comparison with native sarcoplasmic reticulum, we also investigated the effect of CPA on the Na^+,K^+-ATPase activity in a native membrane, without prior purification. The Na^+,K^+-ATPase of brain microsomes (20) was 0.37 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ in the absence of CPA, representing 79% of the total ATPase activity; the corresponding values were 0.38 and 0.43 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ in the presence of 100 and 1000 nmol of CPA, respectively, indicating no inhibition by CPA. The Mg^{2+}-stimulated, Na^+- and ouabain-independent component of ATP hydrolysis in brain microsomes was 0.10 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ in the absence of CPA and remained essentially unchanged (0.12 $\mu mol \cdot mg^{-1} \cdot min^{-1}$) in the presence of 1000 nmol/mg CPA.

Cyclopiazonic acid was also essentially without effect, even at concentrations as high as 10 μmol/mg protein on the Mg^{2+}-stimulated ATPase of T-tubules and surface membranes (Table III), assayed at pH 6.8 and 7.8, in the presence of 4 mM EGTA, that lowered the Ca^{2+} concentration to $\simeq 10^{-9}$ M. Under these conditions the Mg^{2+}-stimulated, Ca^{2+}-independent ATPase activity of the surface membrane preparation represented more than 80% of the total activity measured in the presence of 5 mM $MgSO_4$ and 0.1 mM $CaCl_2$. By contrast,

TABLE II

The effect of CPA on the hydrolysis of ATP by the Na^+,K^+-ATPase, H^+,K^+-ATPase, mitochondrial F_1-ATPase, and the Ca^{2+}-ATPase of erythrocytes

The ATPase activities were tested by the coupled-enzyme assay at the indicated ATP and CPA concentrations, as described under "Experimental Procedures." Each value is the average of 2–10 independent measurements. The Ca^{2+}-dependent ATPase activity of the sarcoplasmic reticulum Ca^{2+}-ATPase determined in parallel assays, under the assay conditions used for the various enzymes, was inhibited by 59–89% in the presence of 100 nmol of CPA/mg protein. The specific activities of the sarcoplasmic reticulum Ca^{2+}-ATPase tested in the absence of CPA ranged from 1.4 to 1.8 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ under the conditions used for the gastric H^+,K^+-ATPase, from 0.95 to 1.5 under the conditions of the Na^+,K^+-ATPase, and from 2.3 to 3.4 under the conditions of the erythrocyte Ca^{2+}-ATPase. The activity of the sarcoplasmic reticulum Ca^{2+}-ATPase in the absence of CPA was relatively low (0.75 $\mu mol \cdot mg^{-1} \cdot min^{-1}$) in the assay system of mitochondrial F_1-ATPase containing 0.6 mM ATP. The relatively low activity under these conditions may be due to inhibition by free Mg^{2+}, since at 6 mM ATP concentration the activity rose to the usual range (3.85–4.71 $\mu mol \cdot mg^{-1} \cdot min^{-1}$).

CPA	Gastric H^+,K^+-ATPase		Kidney Na^+,K^+-ATPase		Mitochondrial F_1-ATPase		Erythrocyte Ca^{2+}-ATPase	
	2 mM ATP	0.2 mM ATP	3 mM ATP	0.3 mM ATP	6 mM ATP	0.6 mM ATP	5 mM ATP	0.5 mM ATP
nmol/mg protein	$\mu mol \cdot mg^{-1} \cdot min^{-1}$				$\mu mol \cdot mg^{-1} \cdot min^{-1}$			
0	0.14	0.075	2.66	1.53	27.8	6.6	0.61	0.65
100	0.20	0.074	2.75		28.1	6.8	0.60	0.62
200	0.12		3.03		32.2		0.63	
500	0.15		2.81		27.2		0.62	
1000	0.17	0.066	2.71	1.38	30.5	7.3	0.59	0.66

TABLE III

A comparison of the effect of CPA on the phosphohydrolase activities of sarcoplasmic reticulum and surface membrane preparations

The hydrolysis of ATP (2 mM) was measured at pH 6.8 or 7.8, at 25 °C, either in the presence of 0.1 mM $CaCl_2$ (total activity) or in the presence of 4 mM EGTA (Ca^{2+}-independent activity) using the coupled-enzyme assay system, as described under "Experimental Procedures." The Ca^{2+}-dependent ATPase activity was calculated as the difference between the total and the Ca^{2+}-independent hydrolysis rates. The CPA concentration, when indicated, was 100 nmol/mg protein for the sarcoplasmic reticulum and 1000 nmol/mg protein for surface membrane preparations. CPA (1000 nmol/mg protein) *completely* abolished the Ca^{2+}-dependent ATPase activity of sarcoplasmic reticulum, leaving the Ca^{2+}-independent ATPase component unaffected (not shown). The Ca^{2+}-independent ATPase activity of the surface membranes was also tested with 100 and 10,000 nmol of CPA/mg protein, and at 0.2 and 0.02 mM ATP concentration, without significant inhibition (not shown).

	Sarcoplasmic reticulum						Surface membranes and T-tubules					
	pH 6.8			pH 7.8			pH 6.8			pH 7.8		
	−CPA	+CPA	% change	−CPA	+CPA	% change	−CPA	+CPA	% change	−CPA	+CPA	% change
	$\mu mol \cdot mg^{-1} \cdot min^{-1}$						$\mu mol \cdot mg^{-1} \cdot min^{-1}$					
Total activity	2.62 ±0.03	0.38 ±0.09	−85	2.51 ±0.10	0.60 ±0.22	−76	9.29 ±0.31	8.44 ±0.18	−9	9.30 ±1.29	8.40 ±1.14	−9
Ca^{2+}-dependent activity	2.46 ±0.03	0.20 ±0.07	−92	2.33 ±0.08	0.44 ±0.22	−81	0.61 ±0.26	−0.10 ±0.16	−100	0.93 ±0.41	0.15 ±0.39	−84
Ca^{2+}-independent activity	0.16 ±0.001	0.18 ±0.02	+12	0.18 ±0.02	0.16 ±0.001	−12	8.68 ±0.44	8.54 ±0.16	−2	8.37 ±1.00	8.25 ±0.93	−1

in the sarcoplasmic reticulum vesicles the Ca^{2+}-independent ("basal") ATPase activity is only about 5% of the total activity. The small Ca^{2+}-activated component of ATP hydrolysis in the surface membrane preparations was inhibited by CPA and may represent slight admixture of sarcoplasmic reticulum elements. The Ca^{2+}-independent basal ATPase activity of sarcoplasmic reticulum was resistant to cyclopiazonic acid, consistent with its proposed identity with the Mg^{2+}-ATPase of the surface membranes and T-tubules (16, 31, 39). Contaminating surface membrane vesicles may also account for the Ca^{2+}-independent component of acetylphosphate and *p*-nitrophenylphosphate hydrolysis that was also unaffected by cyclopiazonic acid (Table I). The purified preparation of Ca^{2+}-ATPase obtained as described by Meissner *et al.* (15) did not contain a detectable Ca^{2+}-insensitive component, and the inhibition of the Ca^{2+}-stimulated ATPase by CPA was somewhat greater than in native sarcoplasmic reticulum vesicles.

These observations establish cyclopiazonic acid as a highly selective, high affinity inhibitor of the Ca^{2+}-ATPase of sarcoplasmic reticulum. The mechanism of inhibition by CPA is likely to involve a feature of the sarcoplasmic reticulum Ca^{2+} pump that is distinct from the other P and F type ATPases tested in this study. Covalent labeling of the Ca^{2+}-ATPase with a radioactive form of cyclopiazonic acid may help in the identification of the region of the molecule where CPA exerts its striking effect on the conformational transitions related to Ca^{2+} transport. CPA may also be useful to assess the contribution of sarcoplasmic reticulum Ca^{2+}-ATPase to the Ca^{2+} regulation and ATPase activity of intact and permeabilized living cells and membrane preparations that contain a mixture of different ATPases.

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Note Added in Proof—Cyclopiazonic acid also inhibits the Ca^{2+}-ATPase of rabbit cardiac sarcoplasmic reticulum. Therefore, the structural features involved in the inhibition are shared by the fast

and slow isoenzymes of the Ca^{2+}-ATPase but are distinct from the other P and F type ATPases tested in this study.

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